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Association between cytokine profile and transcription factors produced by T-cell subsets in early- and late-onset pre-eclampsia

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Summary

Pre-eclampsia (PE) is an obstetric pathology characterized by abnormal activation of the innate and adaptive immune systems dependent on the imbalance of T helper subsets. The present study aimed to evaluate the gene and protein expression of T helper type 1 (Th1)/Th2/Th17/regulatory T (Treg) cell transcription factors in peripheral blood lymphocytes from pregnant women with PE employing quantitative RT-PCR and flow cytometry techniques, as well as the cytokine profile produced by these $CD4^+$ T-cell subsets in the plasma of pregnant women with PE, classified as early-onset PE ($n = 20$), late-onset PE ($n = 20$) and normotensive pregnant women ($n = 20$). Results showed a higher percentage of $CD4^+$ T cells expressing the RORc transcription factor (Th17) and a lower percentage of cells expressing FoxP3 (Treg) in women with early-onset PE compared with late-onset PE and normotensive groups. A lower gene expression of GATA-3 transcription factor was detected in cells of women with early-onset PE compared with the late-onset PE group. Endogenous plasma levels of interleukin-6 (IL-6), IL-17 and tumour necrosis factor-α were significantly higher in the early-onset PE group than in the late-onset PE and normotensive groups, whereas IL-4 (Th2 profile) and IL-22 (Th17 profile), were not significantly different between the studied groups. The endogenous levels of transforming growth factor-β and IL-10 were significantly lower in the pre-eclamptic than in the normotensive groups of the same gestational age, with a significant difference between early- and late-onset PE. The results show that in women with PE there is an imbalance between inflammatory and anti-inflammatory profiles in $CD4^+$ T-cell subsets, with polarization to Th17 profiles in the early-onset PE, considered as the severe form of PE.

Keywords: cytokines; regulatory T cells; reproductive immunology; T cells; transcription factors.

Introduction

Pre-eclampsia (PE) is a specific syndrome of human pregnancy that affects 2–10% of women[1] and is responsible for a high proportion of maternal and fetal morbidity and mortality, especially in low- and middle-income countries.[2,3] The clinical diagnosis is based on the development of hypertension (blood pressure $\geq$ 140/90 mmHg) and proteinuria ($\geq$ 300 mg/24 hr) that occurs from the 20th week of pregnancy.[4] Other maternal

Abbreviations: APC-Cy7, allophycocyanin conjugated to cyanine 7; APC, allophycocyanin; BB515, bright blue fluorochrome 515; FoxP3, forkhead box P3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GATA-3, GATA binding protein 3; IFN-γ, interferon-γ; IL, interleukin; NT, normotensive; PE, pre-eclampsia; PerCP-Cy5·5, pyridine protein chlorophyll conjugated with cyanine dye; RORc, retinoic acid-related orphan receptor C; RT-qPCR, real-time quantitative polymerase chain reaction; T-bet, T box transcription factor; TGF-β_1, transforming growth factor β_1; Th1, T helper type 1; TNF-α, tumor necrosis factor α; Treg, regulatory T cell

dysfunctions are also related to PE, such as renal insufficiency, liver involvement, neurological or haematological complications, utero–placental dysfunction or fetal growth restriction.[5,6]

Clinically, PE is classified as mild or severe, according to patient signs and symptoms[1] and as early-onset and late-onset PE, depending on whether clinical manifestations occur before or after 34 weeks of gestation, respectively.[7,8] According to Huppertz[8] early- and late-onset PE are two entities that differ in aetiology and in disease manifestation. Early-onset PE is associated with placental dysfunction, abnormal uterine and umbilical artery Doppler evaluation, fetuses with growth restriction, intrauterine fetal or maternal complications[9,10] and a higher recurrence rate.[11] On the other hand, late-onset PE is often associated with a normal or slightly increased uterine resistance index, low rate of fetal compromise and more favourable perinatal outcomes.[2,12]

In PE, excessive activation of peripheral blood leucocytes is associated with exaggerated innate and adaptive immune responses that may interfere with normal pregnancy progression.[13] This immunological disturbance seems to result from an exacerbated activation of the maternal inflammatory response characterized by overproduction of pro-inflammatory cytokines such as interferon-γ (IFN-γ), interleukin-1β (IL-1β), IL-6 and tumour necrosis factor-α (TNF-α)[14–16] and lower concentrations of the regulatory cytokine IL-10 in the circulation or produced endogenously by monocytes from peripheral blood.[17,18]

During the early stages of gestation, a balance is observed in the adaptive immunity cells that comprise $CD4^+$ T cells, the major population involved in the cell-mediated immune response. These cells are activated by antigen-presenting cells and proceed to clonal expansion and cytokine secretion.[19] The cytokine profile secreted at this stage will determine cell differentiation into any of the several subsets of T helper cells and so define the type of immune response generated.[20] Depending on the changes in different cytokine-induced microenvironments $CD4^+$ T lymphocytes can differentiate into the T helper type 1 (Th1), Th2, Th17 or regulatory T (Treg) subsets performing inflammatory, regulatory or suppressor functions. The generation of Th1 cells occurs in the presence of the cytokines IFN-γ, IL-12 and IL-18, which are potent inflammatory mediators for adaptive immune response activation.[21] These cells are characterized by the transcription factor T box transcription factor (T-bet) and expression of IFN-γ and IL-2.[22] The presence of IL-4 together with the transcription factor GATA-3 are responsible for differentiating Th2 cells that express a unique set of cytokines, including, IL-4, IL-5 and IL-13.[22] Th17 cells are also a lineage of $CD4^+$ T cells, which are differentiated in the presence of IL-6 and transforming growth factor-β (TGF-β). Furthermore, these cells are characterized by expressing the transcription factor retinoic acid-related orphan receptor C (RORc) and the cytokine IL-17 production.[23] This subset plays a critical role in the induction of inflammation and in the pathogenesis of autoimmune diseases and tissue rejection.[22,24] On the other hand, Treg cells, essential for maintaining pregnancy and regulating inflammation, are differentiated in the predominant environment of TGF-β[23] together with the transcription factor Forkhead box P3 (FoxP3), the master gene for the differentiation to Treg cells.[25,26] These immune cells express specific anti-inflammatory cytokines such as IL-10 and TGF-β, which dampen an excessive effector immune response.[23,27,28] These factors are activated when a cytokine binds to the specific receptor. In addition to these factors, other DNA binding proteins and epigenetic changes are activated, allowing adequate transcription of genetic information to occur.[29] Hence, determination of $CD4^+$ T-cell subsets through their respective transcription factors is important to understand the role of these T-cell subsets in several pathologies.

The literature shows that T helper subsets, such as Th1, Th2, Th17, and Treg, participate in the pathogenesis and progression of PE, with a predominance of the production of Th1 and Th17 cytokines.[30,31] According to Figueiredo & Schumacher[23] Th17 and Treg cells form complex and dynamic networks to maintain homeostasis. These cells interact with other cell types to modulate the desired immune response during pregnancy. However, in PE, this complex does not occur properly because there is an imbalance between the inflammatory and anti-inflammatory profiles of $CD4^+$ T-cell subsets.

Darmochwal-Kolarz *et al.* and Santner-Nanan *et al.*[31,32] reported a decrease in regulatory T cells and an increase in Th17 profile cells in the peripheral blood of women with PE compared with normal pregnant women, suggesting that this imbalance is responsible for the activation of the inflammatory response in this pathology.

The activation of innate immunity cells detected in PE results in the production of inflammatory cytokines that activate cells of adaptive immunity, leading to increased inflammation. The aim of this study is to evaluate whether the subsets of $CD4^+$ T cells (Th1, Th2, Th17 and Treg) and the cytokine profile produced by these cells may differentiate between early- and late-onset PE. This knowledge may contribute to a better understanding of the involvement of adaptive immunity in the pathophysiology of this important gestational pathology.

Materials and methods

Subjects

The study consisted of 60 primiparous women without previous history of hypertension or obstetric and medical complications, admitted to the Obstetric Unit of Botucatu Medical School, Sao Paulo State University, Botucatu, SP,

Brazil between September 2015 and September 2016. Forty women were diagnosed with PE, defined as a persistent elevated blood pressure value of 140 × 90 mmHg and proteinuria (≥ 300 mg in urine collected during 24 hr) after the 20th week of gestation.[4] Women with PE were classified according to the onset of clinical manifestations of the disease at moment of diagnosis as early-onset PE (< 34 weeks of gestation, $n = 20$) and late-onset PE (≥ 34 weeks of gestation, $n = 20$), according to the criteria suggested by Huppertz[8] A group of 20 normotensive primiparous women with an uncomplicated pregnancy and who remained normotensive (NT) and non-proteinuric until the end of gestation were recruited as controls and matched for gestational age at time of sampling with the groups of women with PE. Gestational age was calculated from the last menstrual period and confirmed by early (< 12 weeks gestation) ultrasound examination. Proteinuria in 24-hr urine was measured by a colorimetric method, the Technicon RAXT automation system, in the Clinical Laboratory, Botucatu Medical School, Botucatu, SP, Brazil. Exclusion criteria included multiple gestation, previous PE, illicit drug use and pre-existing medical conditions such as diabetes, cancer, chronic hypertension, acute infectious diseases, and cardiovascular, autoimmune, renal and hepatic diseases. The study was approved by the Research Ethics Committee of Botucatu Sao Paulo State University (UNESP) Medical School (CAAE Protocol number: 43467315 3 0000 5411), and written informed consent was obtained from all women involved in the study. For pregnant women aged below 18 years the written informed consent was obtained from their parents or guardians.

Blood sampling

The whole blood for evaluation of T-cell subsets and determination of cytokines from pregnant women with PE was collected at the time of disease diagnosis, and from NT pregnant women at the time they were matched for gestational age with women with PE. Blood samples (10 ml) were collected by venepuncture from the antecubital vein and were put into a sterile plastic tube containing 10 U/ml EDTA (Becton Dickinson-BD Vacutainer; BD Biosciences, Franklin Lakes, NJ). After centrifugation for 10 min at 3000 ***g***, the obtained plasma was removed and aliquots were stored at −80° until the time of cytokine determination.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells were isolated by density gradient centrifugation on Ficoll-Paque Premium (density = 1·077) (GE Healthcare Bio-Sciences, Uppsala, Sweden) as described previously.[15] The obtained mononuclear cell-rich ring was washed twice with RPMI-1640/HEPES tissue-culture medium (LGC Biotechnology, Sao Paulo, SP, Brazil) with centrifugation between washes at 300 ***g*** for 10 min. After this procedure, the cells were resuspended in RPMI-1640/HEPES culture medium (LGC Biotechnology) supplemented with 10% inactivated fetal bovine serum (complete RPMI). For identification of the mononuclear cells, 50 µl of the mononuclear cell suspension was incubated for 10 min at 37° with 450 µl of 0·02% neutral red solution. The cell concentration was adjusted to 1×10^6 viable cells/ml, and the cells were distributed (1 ml/well) in 24-well flat-bottomed plates (Falcon, Corning Incorporated-Life Sciences, Durham, NC) and incubated at 37°, in a 5% CO_2 atmosphere for 90 min. Non-adherent cells were obtained by washing the plate wells with RPMI-1640/HEPES culture medium (LGC Biotechnology). Cell viability as determined by 0·2% Trypan blue dye exclusion was > 95% in all experiments. The cell concentration was adjusted to 2×10^5 viable cells/ml for T-cell subset characterization by flow cytometry.

Analysis of the expression of transcription factors in T lymphocytes by flow cytometry

Expression of intracytoplasmic transcription factors for Th1 (T-bet), Th2 (GATA-3), Th17 (RORc) and Treg (FoxP3) cells was evaluated soon after blood collection (endogenous expression). The cell concentration was adjusted to 2×10^5 cells/ml and cells were distributed in Falcon cytometer tubes (BD Biosciences). The cells were incubated with BD Biosciences antibodies, with respective fluorochromes: anti-CD3 (phycoerythrin-Cy7), anti-CD4 [allophycocyanin (APC)], anti-CD25 (APC-H7) and anti-CD127 (BB515) for 30 min in the dark. After centrifugation the cells were washed with wash buffer (BD Biosciences) and centrifuged again at 400 ***g*** for 5 min. Then, the cells were fixed and permeabilized using perm buffer (BD Biosciences) for incubation with specific antibodies (BD Biosciences) labelled with specific fluorochromes for the intracellular proteins T-bet [peridinin chlorophyll protein (PerCP) -Cy5·5], GATA-3 (phycoerythrin), RORc (phycoerythrin) and FoxP3 (phycoerythrin) for 30 min in the dark and at room temperature. For the delineation of the gates, control tubes were incubated with specific isotype antibodies for each fluorochrome (phycoerythrin-Cy7, APC, APC-H7, BB515, PerCP-Cy5·5 and phycoerythrin) in each test performed. The gating strategy to analyse Th1, Th2, Th17 and Treg subsets was performed inside singlets > debris > $CD3^+$ lymphocytes > $CD4^+$ lymphocytes. Additionally, Treg cell analysis was performed inside a $CD25^+$ $CD127^{low}$ gate. In the case of the measurements, 30 000 events for each sample were required on a FACSCanto™ II flow cytometer (BD Biosciences) with FACSDIVA software (BD Biosciences). The results were analysed in the software FLOWJO, version vX.10·6 (FlowJo, LLC, Ashland, OR).

Evaluation of the expression of transcripts related to transcription factors of CD4$^+$ T-cell subsets by quantitative real-time PCR

The non-adherent peripheral blood mononuclear cells were evaluated for the expression of genes encoding T-bet, GATA-3, RORc and FoxP3 proteins at the transcriptional level. Total RNA was extracted from 2×10^5 viable cells using the Total RNA Purification Kit (Norgen Biotek Corp., Thorold, Canada) according to the manufacturer's protocol, and the quantitative RT-PCR (RT-qPCR) was performed as described previously.[33] Briefly, isolated RNA was treated with DNAse I Amp Grade (Invitrogen, Carlsbad, CA). Subsequently, the synthesis of cDNA was conducted using ImProm-II™ Reverse Transcription System, according to the manufacturer's protocol. The RT-qPCR was made using RT GoTaq-qPCR Master Mix (Promega, Madison, WI) and the primer sequences used in this study are listed in Table 1. Each reaction was set in duplicate and the conditions for the RT-qPCR were as follows: initial denaturation at 96° for 2 min and then 40 cycles at 95° for 15 seconds and 60° for 60 seconds, followed by a melting curve. Expression values of the analysed transcripts were normalized to that of the enzyme-encoding glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH). The calculation of the differential expression of selected genes was carried out by the data processing method compared with a standard curve.[34] To analyse relative gene expression, we standardized the RNA expression levels in all samples to that of a single RNA sample, which was set to a value of 100.

Detection of cytokines related to Th1, Th2, Th17 and Treg subsets by ELISA

Cytokine concentrations in plasma were determined by ELISA using Quantikine ELISA kits (R&D Systems, Minneapolis, MN) for IFN-γ and TNF-α (Th1), IL-4 (Th2), IL-22 and IL-6 (Th17) and IL-10 and TGF-β_1 (Treg). Assay sensitivity limits for IFN-γ, IL-4, IL-6, IL-10, IL-22, TGF-β_1 and TNF-α were 15·6 pg/ml, 10 pg/ml, 0·70 pg/ml, 3·9 pg/ml, 0·7 pg/ml, 1·7 pg/ml and 0·5 pg/ml, respectively. A high sensitivity commercial kit obtained from Affymetrix eBioscience (San Diego, CA) was employed for IL-17 cytokine (Th17) determination with sensitivity limit of 0·01 pg/ml.

Statistical analysis

The results were evaluated using parametric or non-parametric methods, according to the PRISM statistical program (Graph Prism for Windows, version 6·01; GraphPad, San Diego, CA). The clinical characteristics of pregnant women were analysed by Kruskal–Wallis test, followed by multiple comparisons by Dunn's test. Analysis of the subsets of CD4$^+$ T cells and cytokines was evaluated by analysis of variance, followed by multiple comparisons by the Tukey's test. The significance level adopted for all the tests was 5% ($P < 0{\cdot}05$).

Results

Clinical characteristics

There were no significant differences with respect to maternal age and gestational age between pre-eclamptic and normotensive corresponding groups of pregnant women (Table 2). Systolic and diastolic blood pressures were significantly higher in both pre-eclamptic groups compared with the normotensive ones. Proteinuria levels were higher in the early-onset PE group than in the late-onset and normotensive groups. In women with late-onset PE these values were significantly higher than in the normotensive women with the same gestational age.

Expression of transcription factors in CD4$^+$ T-cell subsets

Results of Fig. 1 show the analysis of CD4$^+$ T-cell subsets of inflammatory and anti-inflammatory profiles in the

Table 1. Primers for transcription factors and GAPDH

Gene	Sequence (5′–3′)	GenBank
FoxP3	Forward primer: (614) CAGGAAGGACAGCACCCTTT (633) Reverse primer: (726) GGAAGTCCTCTGGCTCTTCG (707)	NM_014009
GATA-3	Forward primer: (174) CTCTTCGCTACCCAGGTGAC (193) Reverse primer: (269) ACGACTCTGCAATTCTGCGA (250)	NM_001002295.1
RORc	Forward primer: (363) CATGTCCCGAGATGCTGTCA (382) Reverse primer: (473) GGTTCCTGTTGCTGCTGTTG (454)	NM_005060·3
T-bet	Forward primer: (906) GGATGCGCCAGGAAGTTTCA (925) Reverse primer: (993) TGGAGCACAATCATCTGGGT (974)	NM_013351
GAPDH	Forward primer: (684)CGTGGAAGGACTCATGACCA(703) Reverse primer: (801)GGCAGGGATGATGTTCTGGA(782)	NM_002046.4

Table 2. Characteristics of pregnant women with pre-eclampsia and normotensive pregnant women

	Groups			
	Pre-eclampsia		Normotensive	
Parameters	< 34 weeks n = 20	≥ 34 weeks n = 20	< 34 weeks n = 10	≥ 34 weeks n = 10
Age (years)	28 (14–40)	24 (16–43)	25 (18–38)	26 (19–37)
Gestational age (weeks)	29 (26–33)	37 (34–40)	30 (26–33)	37 (34–40)
Systolic blood pressure (mmHg)	160[1] (140–200)	155[1] (140–180)	105 (95–110)	100 (95–110)
Diastolic blood pressure (mmHg)	110[1] (90–140)	100[1] (90–120)	65 (60–70)	60 (60–70)
Proteinuria (mg/24 hr)	3105[2] (300–26 052)	535[3] (300–10 800)	< 300	< 300

Results are expressed as the median (range).
[1](P < 0·01) versus mormotensive (NT) < 34 weeks, NT ≥ 34 weeks.
[2](P < 0·01) versus NT < 34 weeks, NT ≥ 34 weeks, pre-eclampsia ≥ 34 weeks.
[3](P < 0·05) versus NT ≥ 34 weeks (Kruskal–Wallis test).

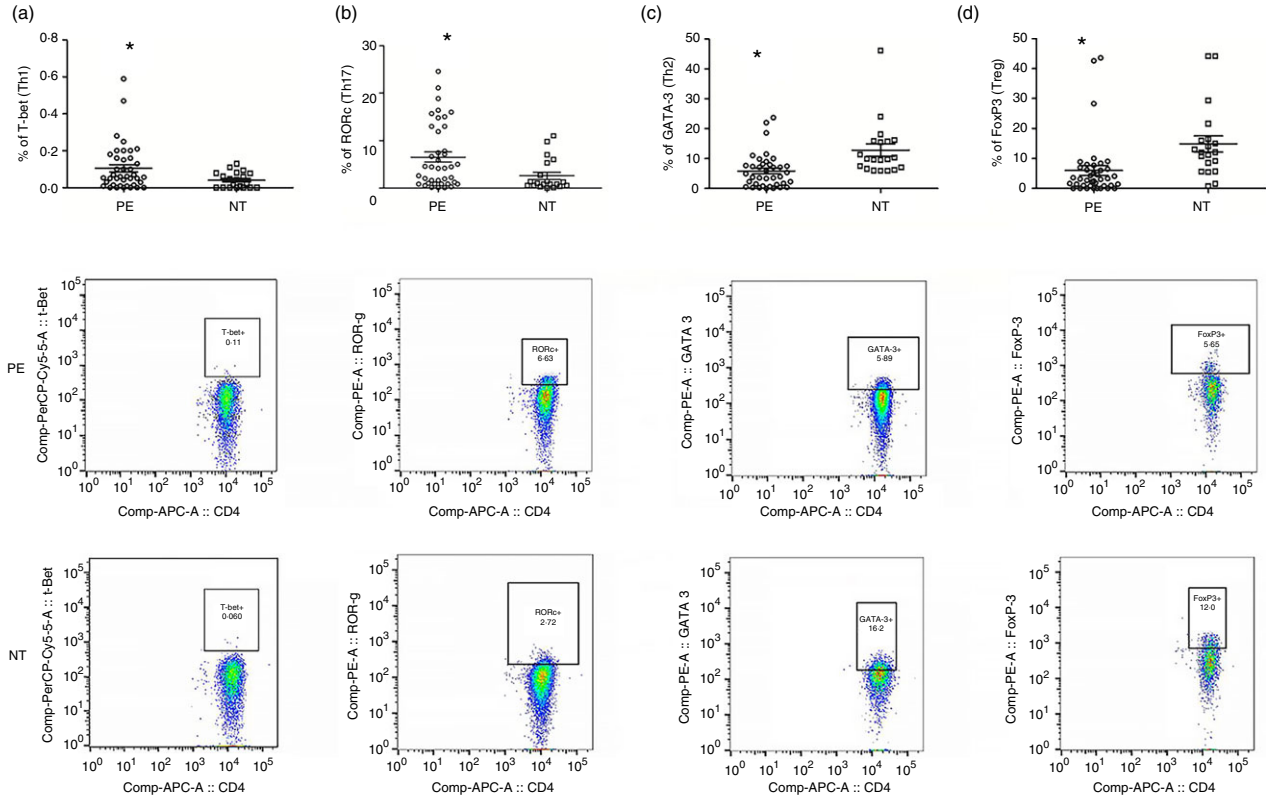

Figure 1. Expression of transcription factors in CD4$^+$ T-cell subsets. Percentage of lymphocytes expressing intracellular transcription factors of inflammatory and anti-inflammatory profiles in pregnant women with pre-eclampsia (PE; n = 40) and normotensive pregnant women (NT; n = 20) by flow cytometry and representative dot plots of each transcription factor. (a) T-bet. (b) RORc. (c) GATA-3. (d) FoxP3. Results expressed as mean ± SD. *(P < 0·05) versus NT (Student's t-test). [Color figure can be viewed at wileyonlinelibrary.com]

groups of pregnant women with PE and the normotensive pregnant women. Representative dot plots of the transcription factors from T-cell subsets were also included. Evaluation of the inflammatory profiles showed that the percentage of cells expressing the T-bet transcription factor, corresponding to the Th1 profile, was significantly higher in the PE group than in the normotensive group (Fig. 1a). Similar results were observed in relation to the Th17 profile, represented by a significant increase in the percentage of cells expressing RORc (Fig. 1b) in women with PE compared with the control group. In the anti-inflammatory profiles for Th2 and Treg, the percentages of lymphocytes expressing GATA-3 (Fig. 1c) or FoxP3 (Fig. 1d), respectively, were significantly lower in

the group of women with PE compared with the normotensive group.

Th17 profile was increased in the early-onset PE

Th1 and Th17 inflammatory profiles were analysed according to the classification of pregnant women in early-onset PE (< 34 weeks of gestation) and late-onset PE (≥ 34 weeks of gestation). The percentage of cells expressing T-bet (Fig. 2a) was significantly higher in women with early-onset PE than in normotensive pregnant women with the same gestational age (< 34 weeks). Similar results were observed in late-onset PE in relation to the normotensive group with equivalent gestational age (≥ 34 weeks). The population of $CD3^+$ $CD4^+$ $RORc^+$ Th17 cells (Fig. 2a) was also significantly higher in the early- and late-onset PE groups compared with the corresponding normotensive groups with the same gestational age. A significant increase in the percentage of Th17 cells expressing RORc was detected in the early-onset PE group compared with the late-onset PE group.

Th2 and Treg profiles were decreased in the early-onset PE

The percentage of lymphocytes expressing GATA-3 was significantly lower in early-onset PE when compared with the normotensive pregnant women with equivalent gestational age (Fig. 2c). A similar result was observed in the late-onset PE group in relation to the normotensive group with the same gestational age. The population of $CD3^+$ $CD4^+$ $CD25^+$ $CD127^{low}$ $FoxP3^+$ Treg cells (Fig. 2d) was significantly lower in both early-onset and late-onset PE groups compared with the corresponding normotensive groups. Comparison between the pre-eclamptic groups showed a significant decrease in the percentage of Treg cells expressing FoxP3 in the early-onset PE compared with the late-onset PE group (Fig. 2d).

mRNA levels of the transcription factors T-bet, GATA-3, RORc and FoxP3 from $CD4^+$ T-cell subsets

No significant differences were detected in the gene expression of T-bet in pregnant women with early- and late-onset PE compared with normotensive pregnant women with equivalent gestational age (Fig. 3a). However, higher mRNA levels of RORc were observed in the early-onset and late-onset PE groups than in the normotensive groups with corresponding gestational age. A significant decrease in gene expression of GATA-3 (Fig. 3c) and FoxP3 (Fig. 3d) was detected in lymphocytes from both early-onset and late-onset PE groups compared with the normotensive groups with the same gestational age. The levels of GATA-3 mRNA were significantly lower in early-onset PE than in late-onset PE. Comparison between normotensive groups showed significant differences between the normotensive group with gestational age < 34 weeks and that with gestational age ≥ 34 weeks (Fig. 3c).

Cytokine determination

The plasma concentrations of IFN-γ (Fig. 4a), TNF-α (Fig. 4b), IL-6 (Fig. 4c) and IL-17 (Fig. 4d) were

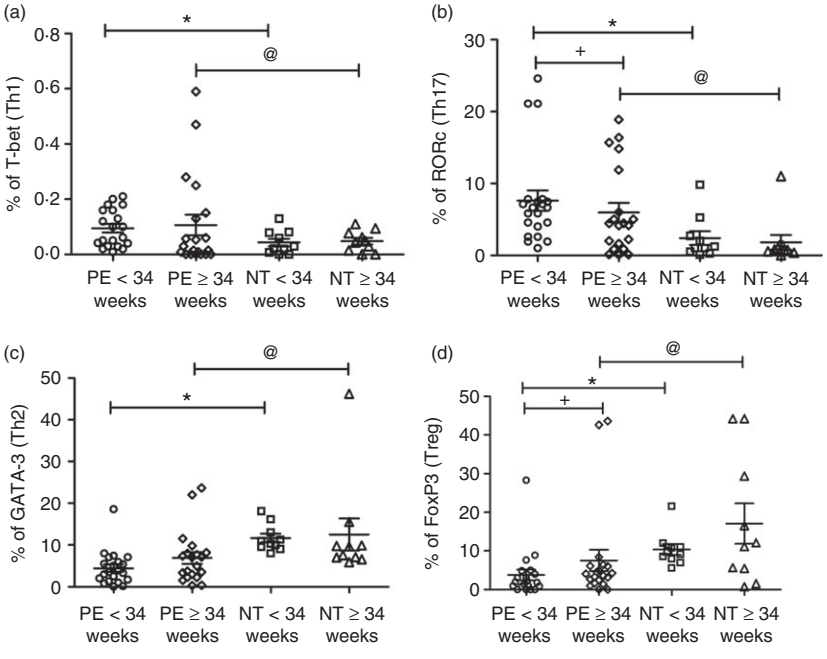

Figure 2. Expression of transcription factors in $CD4^+$ T-cell subsets. Percentage of lymphocytes expressing intracellular transcription factors of inflammatory and anti-inflammatory profiles in pregnant women with early-onset pre-eclampsia (PE < 34 weeks of gestation; $n = 20$), late-onset pre-eclampsia (PE ≥ 34 weeks of gestation; $n = 20$) and normotensive pregnant women (NT < 34 weeks; $n = 10$; NT ≥ 34 weeks; $n = 10$) by flow cytometry. (a) %T-bet. (b) %RORc. (c) % GATA-3. (d) %FoxP3. Results expressed as mean ± SD. *($P < 0{\cdot}05$) versus NT < 34 weeks; $^{@}$($P < 0{\cdot}05$) versus NT ≥ 34 weeks; $^{+}$($P < 0{\cdot}05$) versus PE ≥ 34 weeks (analysis of variance).

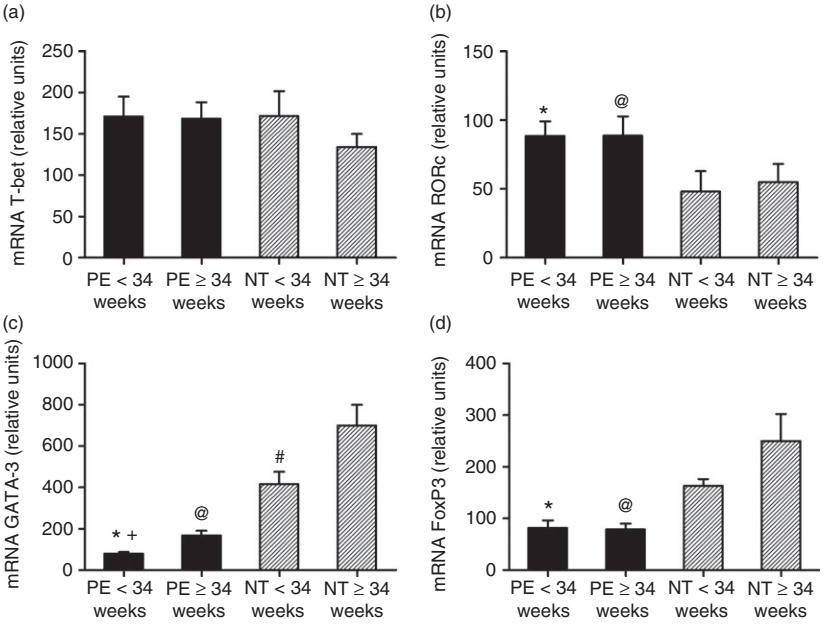

Figure 3. Transcription factors mRNA of pro-inflammatory and anti-inflammatory profiles. mRNA levels of T-bet (a), RORc (b), GATA-3 (c) and FoxP3 (d) in peripheral blood mononuclear cells obtained from pregnant women with early-onset pre-eclampsia (PE < 34 weeks of gestation; $n = 20$), late-onset pre-eclampsia (PE ≥ 34 weeks of gestation; $n = 20$) and normotensive pregnant women (NT < 34 weeks; $n = 10$; NT ≥ 34 weeks; $n = 10$) by RT-qPCR. Results expressed as mean ± SD. *($P < 0{\cdot}05$) versus NT < 34 weeks; $^{@}$($P < 0{\cdot}05$) versus NT ≥ 34 weeks; $^{+}$($P < 0{\cdot}05$) versus PE ≥ 34 weeks; $^{\#}$($P < 0{\cdot}05$) versus NT ≥ 34 weeks (analysis of variance).

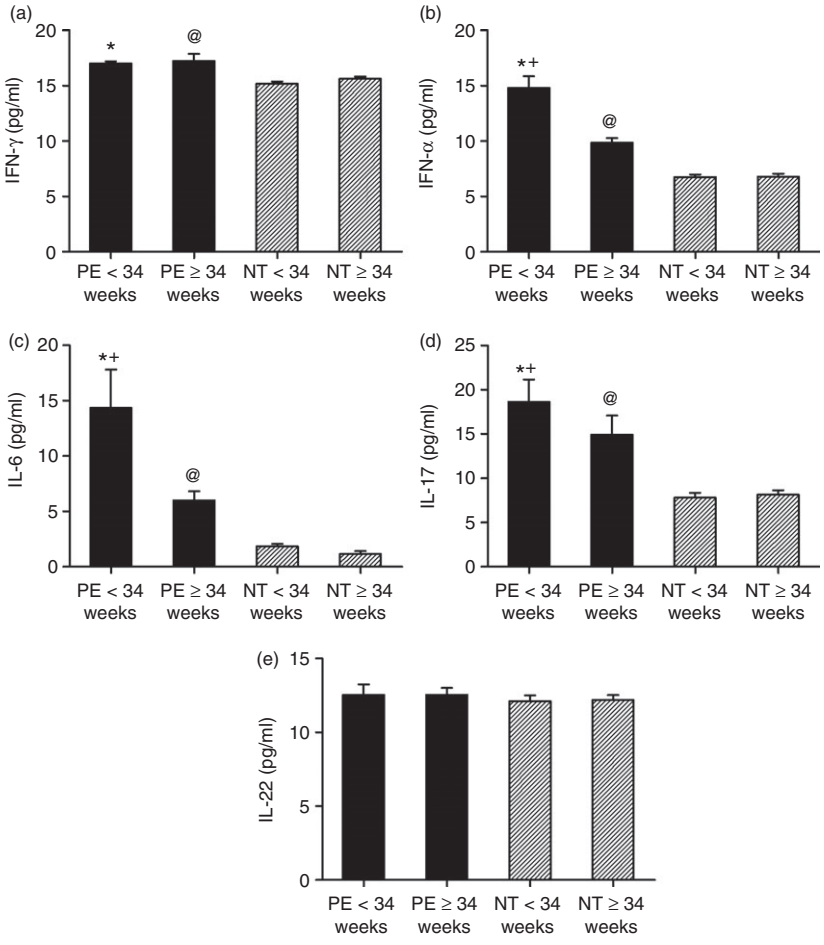

Figure 4. Pro-inflammatory cytokine profile. Protein expression of interferon-γ (IFN-γ) (a), tumour necrosis factor-α (TNF-α) (b), interleukin-6 (IL-6) (c), IL-17 (d) and IL-22 (e) in plasma obtained from pregnant women with early-onset pre-eclampsia (PE < 34 weeks of gestation; $n = 20$), late-onset pre-eclampsia (PE ≥ 34 weeks of gestation; $n = 20$) and normotensive pregnant women (NT < 34 weeks; $n = 10$; NT ≥ 34 weeks; $n = 10$) detected by ELISA. Results expressed as mean ± SD. *($P < 0{\cdot}05$) versus NT < 34 weeks; $^{@}$($P < 0{,}05$) versus NT ≥ 34 weeks; $^{+}$($P < 0{\cdot}05$) versus PE ≥ 34 weeks (analysis of variance).

significantly higher in women with PE than in the normotensive groups with the corresponding gestational age. When the two groups of early-onset and late-onset PE were compared, TNF-α, IL-6 and IL-17 levels were significantly higher in the early-onset PE group. The plasma IL-22 (Fig. 4e) and IL-4 (Fig. 5a) levels showed no

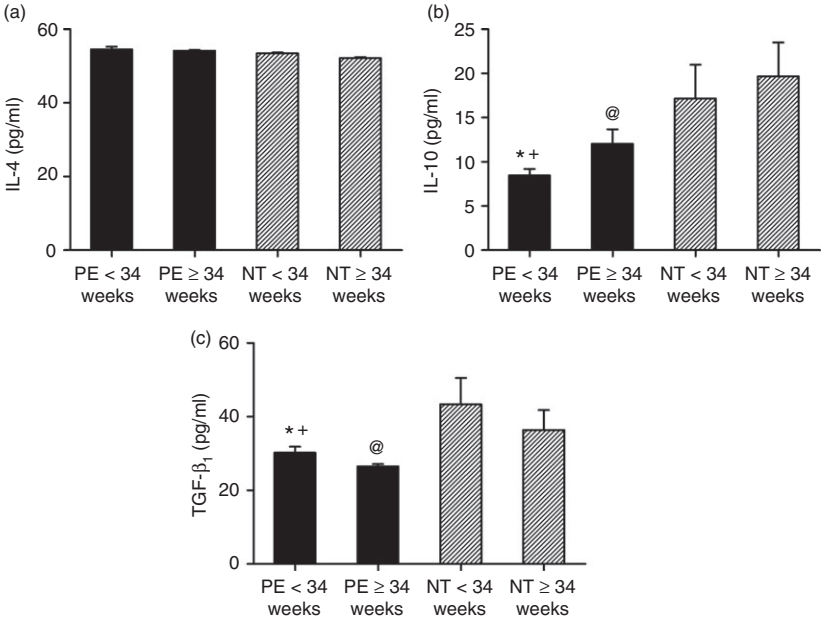

Figure 5. Anti-inflammatory cytokine profile. Protein expression of interleukin-4 (IL-4) (a), IL-10 (b) and transforming growth factor-β_1 (TGF-β_1) (c) in plasma obtained from pregnant women with early-onset pre-eclampsia (PE < 34 weeks of gestation; $n = 20$), late-onset pre-eclampsia (PE ≥ 34 weeks of gestation; $n = 20$) and normotensive pregnant women (NT < 34 weeks; $n = 10$; NT ≥ 34 weeks; $n = 10$) detected by ELISA. Results expressed as mean ± SD. $^{*}(P < 0{\cdot}05)$ versus NT < 34 weeks; $^{@}(P < 0{\cdot}05)$ versus NT ≥ 34 weeks; $^{+}(P < 0{\cdot}05)$ versus PE ≥ 34 weeks (analysis of variance).

significant differences between the normotensive and pre-eclamptic groups.

Endogenous levels of IL-10 (Fig. 5b) and TGF-β_1 (Fig. 5c) were significantly lower in early-onset and late-onset PE groups than in normotensive ones with corresponding gestational age. Evaluation between the pre-eclamptic groups showed respectively lower IL-10 (Fig. 5b) and higher TGF-β_1 (Fig. 5c) concentrations in early-onset PE. There were no differences between the normotensive groups in relation to all the cytokines evaluated.

Discussion

The present study characterized the subsets of $CD4^+$ T lymphocytes (Th1, Th2, Th17 and Treg) and the cytokine profile produced by these cells in the peripheral blood of pregnant women with PE. The results demonstrated that lymphocytes are endogenously polarized to the Th1 and Th17 inflammatory profiles and show a decrease in Th2/Treg anti-inflammatory profiles. In addition, increased production of the pro-inflammatory cytokines IFN-γ, IL-6, IL-17 and TNF-α, as well as decreased levels of anti-inflammatory cytokines IL-10 and TGF-β_1, were found. The levels of IL-4 and IL-22 showed no significant differences between the groups studied. These results confirm previous evidence of exacerbated activation of circulating leucocytes in these patients[14,15,35] and agree with reports showing a decrease in the proportion of circulating Treg cells and an increase in Th17 cells in women with PE.[31,32,36–39]

In PE, T helper cell subsets have been evaluated by gene expression of transcription factors for these cells,[27,40] or by the protein expression of these factors.[31,32,38] In the present study, the gene and protein expression of Th1/Th2/Th17/Treg-specific transcription factors in peripheral blood lymphocytes from pregnant women with PE were determined, using RT-qPCR and flow cytometry techniques, respectively, as well as the cytokine profile produced by these $CD4^+$ T-cell subsets in the plasma of pregnant women with PE. Increased Th1/Th17 inflammatory profiles and decreased Th2/Treg anti-inflammatory profiles in women with PE showed imbalance between $CD4^+$ T-lymphocyte subsets, evidenced by increased gene and protein expression of T-bet and RORc intracytoplasmic transcription factors and by lower gene and protein expression of GATA-3 and FoxP3 in women with PE. Recently, Gharesi-Fard *et al.*[41] evaluated the gene expression of these transcription factors in decidua and chorionic villi of placentas from pregnant women with PE and normotensive pregnant women by RT-PCR. The authors detected a decrease in expression of FoxP3 and GATA-3 and an increase of T-bet in the decidua, whereas in the chorionic samples, FoxP3 expression was decreased and RORc increased. The similarity between our results and those of these authors suggests that the imbalance of these subsets in the placenta is also observed in the circulating cells of women with PE.

Studies on Th17 cells involved in the pathogenesis of PE detected an increase in the percentage of these cells in the peripheral blood of patients with severe PE compared with healthy pregnant women, suggesting that hyper-regulation of Th17 immunity may contribute to the development of severe cases of PE.[31] On the other hand, Treg cells are considered to play a crucial role in the implantation of embryos and the maintenance of maternal immune tolerance to the fetus.[42] Zhang *et al.*[43] demonstrated that the percentage of Treg cells defined as $CD4^+$ $CD25^+$ $CD127^{low}$

was significantly lower in women with severe PE compared with healthy pregnant women. Hence, this reduction of Treg cells may interrupt maternal immunological tolerance, contributing to the occurrence of PE.

When pregnant women with PE were classified according to the onset of the clinical manifestations of PE, we found that pregnant women with early-onset PE showed a higher percentage of $CD4^+$ T cells expressing the RORc transcription factor and a significant decrease in the percentage of Treg cells expressing FoxP3 in relation to the late-onset PE group, so confirming the severity of the disease before the 34th week of gestation. This Treg/Th17 imbalance may be responsible for the activation of the exacerbated inflammatory response that is more prominent in early-onset than in late-onset PE.[18]

There were no significant differences between the pre-eclamptic groups regarding the gene expression of T-bet, RORc and FoxP3, although GATA-3 expression was lower in pregnant women with early-onset PE. These results suggest that the evaluation of the percentage of these subsets using flow cytometry and the determination of the cytokine profile by ELISA were the most sensitive and adequate techniques to discriminate between pregnant women with early-onset PE and those with late-onset PE in relation to the involvement of adaptive immunity in the disease pathogenesis.

Our study evaluating the specific cytokines of each T-cell subset profile in plasma of pregnant women with PE confirms the polarization of these cells to an inflammatory profile. Hence, high endogenous production of the inflammatory cytokines IFN-γ, TNF-α, IL-6 and IL-17 was observed, as well as lower production of anti-inflammatory cytokines IL-10 and TGF-β_1 detected in the plasma of pregnant women with PE. On the other hand, the levels of IL-4 and IL-22 showed no significant differences between the groups evaluated. The increased levels of IFN-γ produced by women with PE corroborate the results of other authors who detected significant increases of this cytokine in the plasma of pregnant women with PE compared with normotensive pregnant women.[44,45]

Plasma levels of TNF-α were significantly increased in early-onset PE compared with late-onset PE, which confirms previous results obtained in our laboratory.[18] The higher endogenous production of TNF-α in pregnant women with PE is in accordance with the literature[15,46,47] and suggests that the deleterious effects of high TNF-α circulating concentrations may be associated with the most severe forms of PE[18] and with the oxidative stress present in this pathology.[15] It is noteworthy that in the blood of pregnant women with PE there is an exacerbated cytotoxic activity accompanied by an increase in the levels of pro-inflammatory cytokines such as TNF-α and IL-6,[48] in addition to IFN-γ and IL-17.[31,44]

Naive T cells stimulated with TGF-β and IL-6 develop a Th17 response, with expression of IL-17 and IL-22 and are responsible for autoimmunity and gestation loss.[23,28] According to our findings, it was evident that pregnant women with early-onset PE, considered the most severe form of PE, comprised a higher percentage of Th17 cells in the peripheral blood and also significantly higher levels of IL-17 compared with late-onset PE and normotensive women of similar gestational age. These results agree with recent studies showing an increase in circulating levels of IL-17 in pregnant women with PE when compared with healthy pregnant women and non-pregnant women[49] and higher IL-17 concentration in the plasma of pregnant women with severe PE.[43] Considering that pregnant women with early-onset PE develop more severe clinical manifestations than pregnant women with late-onset PE, leading to the excessive systemic inflammatory response characteristic of PE, the high levels of IL-17 produced by Th17 cells may cooperate with other immune mediators to aggravate the inflammation of placental blood vessels, so contributing to the development of PE.[31]

In the present study, the higher levels of IL-6 in pregnant women with early-onset PE compared with the late-onset PE and normotensive groups confirmed data from the literature showing the polarization of the Th17 inflammatory profile through the IL-6 and TGF-β axis.[50] This shift to Th17 was more evident in pregnant women with early-onset PE, which shows a more intense inflammatory state than in women with late-onset PE.[18] The decrease in Th2/Treg anti-inflammatory profile in women with PE could be explained by the predominant inflammatory cytokine environment in these pregnant women. Since IL-1β and IL-6 can induce the development of Th17 from the common ancestral cell of Th17 and Treg cells[51,52] it can be assumed that in PE higher concentrations of these cytokines may direct the differentiation of Treg cells to Th17 cells.[53]

The decreased levels of TGF-β_1 in women with PE compared with increased levels in normotensive pregnant women corroborate the literature[54] showing that the polarization of $CD4^+$ T cells to Treg requires an environment with high levels of TGF-β.[23] Therefore, the Treg profile may be decreased in PE due to the high concentrations of IL-6 produced by cells of innate immunity in pregnant women with PE.[14] As IL-6 secretion suppresses the protective immunoregulatory properties of $CD4^+$ $CD25^+$ Treg cells[55] it is possible that increased levels of IL-6 may regulate Treg cell expansion in this syndrome.[53] Although TGF-β_1 levels were decreased in pregnant women with PE compared with normotensive ones, they were higher in early-onset PE than in late-onset PE, suggesting that the association between higher concentration of IL-6 and TGF-β_1 levels could polarize T cells to the Th17 profile, with higher production of IL-17 in the early-onset PE group.

The concentration of IL-4 in the plasma of women with PE did not show significant difference when

compared with the normotensive groups, corroborating the results of other authors.[48,56] Similarly IL-22 plasma concentrations were not different between the groups studied. Although Zhang *et al.*[43] have reported a significant increase in plasma levels of IL-22 in pregnant women with severe PE compared with the control group, studies on IL-22 in PE are scarce in the literature. Hence, we consider that IL-22 determination in women with PE needs further investigation, to better understand its role in PE.

Plasma levels of IL-10 and the percentage of Treg cells were significantly lower in pregnant women with early-onset PE than in the late-onset PE and normotensive groups, suggesting that both the number and function of this $CD4^+$ subset is impaired in early-onset PE. The highest endogenous concentration of IL-10 detected in plasma of normotensive pregnant women compared with pre-eclamptic groups is in accordance with the findings of the literature.[17,57,58] Hence, the higher production of IL-10 in normotensive pregnant women can be explained by the predominance of an anti-inflammatory Th2 profile in these women, which develops during pregnancy, with predominance of IL-10 over TNF-α, to minimize the deleterious effects of an excessive inflammatory response.

Together,the results of the present study demonstrate for the first time that the deviation of $CD4^+$ lymphocytes to the Th17 profile was more evident in pregnant women with early-onset PE. This cell polarization is demonstrated by a higher percentage of cells expressing RORc and elevated IL-17 production, associated with significantly lower levels of IL-10 and a lower percentage of Treg cells. These results confirm previous studies of the literature showing that the balance between Treg and Th17 cells is deficient in PE.[23,31,32] Considering that maternal adaptation to gestation requires a rigidly controlled interaction between innate and adaptive immunities to allow normal growth and development of the fetal half-graft,[59] the use of agents capable of modulating this interaction might lead to pregnancy success and contribute to a better understanding of the involvement of adaptive immunity in the pathophysiology of PE.

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Author contributions

VRR, MRV, GGR, MLM and PRN performed experiments. VTB and JCP selected pregnant women for the study. VRR, MRV and MTSP conceived the ideas, designed experiments, analysed data and prepared the manuscript.

Disclosures

The authors state that they have no financial or commercial conflicts of interest.

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